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Scientific Officer
A.J. Melaragno, CAPT, MC, USN
Naval Medical R&D Command
Director of Research and Development
Bethesda, MD 20814-50440

Ref: N00014-91-C-0044

Dear Captain Melaragno:

Enclosed in the First Triannual Report for Contract No.: N00014-91-C-0044, which is entitled "Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite". This Report covers the period from Feb. - May, 1991. If you have any questions about the Report or the research, please contact me at 404-952-1660.

Sincerely,

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Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite

First Triannual Report: February - May, 1991

Introduction. Our early efforts on this contract have been devoted to developing key assays and establishing initial model systems for testing. Below we outline the preliminary results of studies testing the effects of stresses encountered during nonfreezing cold injury (e.g., low temperature, acidosis, anoxia, elevated inorganic phosphate levels, and intracellular ionic imbalance) on the model systems. Once we have established the acute effects, we will then begin to investigate the mechanism(s) of irreversible damage induced by long-term exposure to these conditions. In addition, we will then apply the methodology to other the sensitive cell types that are involved in cold injury.

Metabolic studies. We have chosen human red blood cells as the first model for the study of the metabolic consequences of hypothermia and acidosis. Our hypothesis is that low pH and temperatures will synergistically inhibit glycolysis. Phosphofructokinase, the key regulatory enzyme of glycolysis, is inhibited *in vitro* to a greater degree at a given pH as temperature is lowered. This is not simply a Q_{10} effect and is most likely due the increase in the pKa of essential histidine residues as temperature is lowered. That is, as the temperature is lowered, a given degree of protonation of these residues can be achieved at a higher pH. This property of the enzyme has been documented for skeletal muscle homologues *in vitro*. A corollary to our hypothesis is that this *in vitro* property will be operative *in vivo* for the enzyme from a variety of sources, including red blood cells.

We used heat conduction microcalorimetry to measure the rates of total metabolic heat production by red cells as a function of pH and temperature. A two-way matrix of pH and temperature has been tested. For each temperature (i.e., 37, 30, 25, 20, 15, and 10°C) the following external pH's have been tested: 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6. The highest rates of heat production are noted at pH's $\geq$ 8.0 and 37°C. A plot of heat production rates vs. pH for cells at 37°C is sigmoidal, with apparently complete glycolytic inhibition noted at pH $\leq$ 6.8. The residual heat production at acidotic pH (about 40% of total heat) has been ascribed by previous researchers to the pentose phosphate shunt. This heat is approximately equal to that noted in the presence of KF, which is an inhibitor of glycolysis.

In contrast, the pH profile for heat production at lower temperatures (e.g., 20°C) is much flatter, indicating that there may be synergistic inhibition of glycolysis by low temperature and low pH. For example, pH 7.8, which is only slightly inhibitory at 37°C appears to induce much greater inhibition at 20°C.

However, substantiation of this suggestion will require comparison of metabolite levels under the different experimental conditions, and crossover point analysis.

In this manner we will be able to determine if pH-induced glycolytic inhibition accounts for the heat changes we noted in the microcalorimetry experiments, and which regulatory enzymes of glycolysis are affected. We have begun to set up the enzyme-linked fluorometric assays needed for the metabolite analysis. We will soon begin to perform crossover point analysis at each of the key external pH and temperature combinations. In addition, we have started to investigate means by which to measure the intracellular pH in the red blood cells. This parameter tracks fairly closely with external pH. However, it is important to measure these values accurately and make metabolic comparisons based on intracellular pH.

Once we establish conditions under which glycolysis is inhibited -- presumably at phosphofructokinase -- we will then investigate the mechanism for pH-induced inhibition of the enzyme, and the influence of temperature on this process. Protons could affect enzyme activity in three different, but interacting ways: 1) acute effects of protonation on enzyme activity; 2) pH-induced dissociation of active tetramers into inactive dimers; and 3) pH-induced alterations in the association of the enzyme with cell membrane proteins. Our goal will be to discern how these effects work in concert to inhibit phosphofructokinase activity *in vivo*.

Membrane function. The first studies on the effects of ischemia on membrane function have concentrated on determining the influence of inorganic phosphate (Pi) on sarcoplasmic reticulum (SR) function. Whenever muscle is exposed to hypoxia or ischemia, the intracellular concentration of inorganic phosphate, as well as that of protons, increases. Because muscle contraction is regulated by the level of myoplasmic free Ca^{2+}, which is in turn controlled by the SR, we measured the effect of Pi on the Ca^{2+} uptake and release properties of the SR. Pi had no effect on Ca^{2+}-induced Ca^{2+} release from the SR at any Ca^{2+} level. In contrast, Pi had a biphasic effect on Ca^{2+} uptake; at concentrations less than 25 mM, Pi inhibited Ca^{2+} loading, whereas at higher concentrations, Pi increased it. These effects on loading derive from two different processes: 1) Pi inhibits the Ca^{2+}-ATPase and the rate of Ca^{2+} uptake, and; 2) it enhances Ca^{2+} loading by reversibly forming an amorphous calcium phosphate complex within the lumen of the SR. Two manuscripts describing these studies have been prepared and submitted.

The effects of Pi on the SR would be additive with the direct inhibitory effects of Pi on the contractile apparatus itself. Furthermore, acidosis also serves to inhibit both SR and the contractile apparatus. Thus there is a complicated interaction among Pi, pH, the SR, and the contractile apparatus, even under isothermal conditions. The added perturbation of hypothermia increases the level of complexity since it serves to increase sensitivity to lowered pH. And temperature should directly modulate the fluidity of the membrane and thus influence transport, independent of changes in other effectors. As we continue these investigations, we will strive to sort out some of these complex issues to determine how such interacting perturbations ultimately lead to the failure of cells to function (e.g., contract) and to cell death.

Skeletal muscle fiber function. We have determined that lowering pH directly inhibits muscular contraction in skinned fiber preparations. There is a synergistic, inhibitory effect of cold and acidosis on mammalian muscle fibers. For example, about the same degree of reduction in force, which is noted at pH 7.0 at 37°C, is seen at about pH 7.5 at 10°C. In contrast, muscle fibers from

cold-adapted species (e.g., Maine lobsters) do not display sensitivity to acidotic pH unless the temperature is lowered to below about 10°C. We think that these effects can be explained by a similar argument to that proposed above for glycolytic inhibition. That is, for the mammalian systems enhanced pH sensitivity in the cold is most likely due the increase in the pKa of essential amino acid residues of the contractile proteins. Thus, as the temperature is lowered, a given degree of protonation of these residues can be achieved at a higher pH. In contrast, it appears that the contractile proteins in the lobster, which is adapted to the cold, have a lower pKa at low temperatures. Hence they display a pH sensitivity at 10°C that is similar to that seen with mammalian fibers at 37°C.

We are beginning investigations on the mechanism of pH and temperature-induced inhibition of contraction. One model that appears to have great promise is a chimeric muscle fiber that can be constructed using the lobster fiber, into which mammalian proteins have been substituted. When the lobster fibers are held at 37°C, constituent contractile proteins are released from the fiber. This process can be characterized by performing gel electrophoresis of the released proteins. Initial experiments suggest that mammalian proteins can be substituted for the eluted lobster homologues. By characterizing the effects of different substitutions, we will try to determine which proteins in the muscle fiber are responsible for the pH/cold-induced reduction in contractile force

Potential application of antifreeze peptide to cold injuries. We have proposed that antifreeze peptides could potentially have usefulness in preventing frostbite since they lower the freezing temperature of water. However, the levels needed to achieve this effect are prohibitively high (e.g., > 5 mg/ml); both from an economic and, most likely, medical viewpoint. A few reports have shown that additives such as gelatin can greatly enhance the antifreeze activity of glycoproteins obtained from cold-tolerant insects. We have been working with a recombinant antifreeze peptide from winter flounder. To test whether the enhancement noted with the insect proteins could be seen with our recombinant peptide, we have developed assays for measuring freezing point depression with differential scanning calorimetry. We have recently completed validation of the assays methods and will soon begin to test the effects of various additives on the antifreeze activity of the recombinant peptide. If enhanced activity is noted, we will then start to investigate the mechanism of the effect. It could be due to a specific interaction between the peptide and the additive or to a nonspecific, thermodynamic influence of the additive. For example, solutes which were preferentially excluded from the peptide would increase its chemical potential and possibly its activity. Our overall goal from this section of the study is to discover the means by which to obtain the greatest possible freezing point depression activity from the lowest concentration of antifreeze peptide.